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Review

Readthrough compounds for nonsense mutations: bridging the translational gap

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Approximately 10% of all pathological mutations are nonsense mutations that are responsible for several severe genetic diseases for which no treatment regimens are currently available. The most widespread strategy for treating nonsense mutations is by enhancing ribosomal readthrough of premature termination codons (PTCs) to restore the production of the full-length protein. In the past decade several compounds with readthrough potential have been identified. However, although preclinical results on these compounds are promising, clinical studies have not yielded positive outcomes. We review preclinical and clinical research related to readthrough compounds and characterize factors that contribute to the observed translational gap.

Highlights

Approximately 10% of all pathological mutations are nonsense mutations which are responsible for the most severe cases of genetic diseases but for which no treatment regimens are available.

The main strategy for treating nonsense mutations is by enhancing ribosomal readthrough of premature stop codons through the action of readthrough compounds, thus restoring production of full-length protein.

Most preclinical studies on readthrough compounds have yielded positive results, but have been performed in reporter-based assays despite recent scientific developments that have significantly improved disease models, such as patient-derived cells in functional assays.

Clinical trials have yielded disappointing results, and further interpretation of readthrough compound efficacy across clinical trials is challenging because of differences in research design, treatment length, and clinical endpoints.

Characterization of factors that contribute to the translational gap between readthrough compounds in preclinical studies versus clinical trial results is necessary to make sense of nonsense mutation therapy.

The striking gap between preclinical and clinical studies

In the past decade several new therapeutics have been developed for rare hereditary diseases. However, the therapeutic perspective differs to a large extent between patients and is greatly influenced by the type of mutation. Therapy development has proved particularly challenging for nonsense or PTC mutations which change the original amino acid (AA) codon into a stop codon. Nonsense mutations result in aberrant translation and low levels of truncated proteins with loss-of-function characteristics [1,2]. For patients carrying PTC mutations, treatment is based on treating symptoms because no curative treatments are available. Globally, there are roughly 6000 known genetic disorders, of which the best known PTC disease examples are **cystic fibrosis** (**CF**, see Glossary) and **Duchenne muscular dystrophy (DMD)** [3]. Approximately 10% of all pathological mutations are PTC mutations, accounting for a significant number of affected patients [1]. In addition, PTC mutations have been reported in the context of cancer in tumor-suppressor genes such as *PTEN* and *TP53* [4,5]. As such, compounds targeting PTC mutations have broad, disease-encompassing potential.

The most widespread strategy for treating PTC mutations is aimed at enhancing ribosomal **readthrough** of premature stop codons by incorporating an AA at the PTC site, thus restoring the production of a full-length, functional protein. Since the discovery of aminoglycosides as the first class of compounds that stimulate ribosomal readthrough by slowing down the ribosome, thereby increasing the chance of inducing incorporation of AAs at the PTC site [6], >30 therapeutic readthrough compounds have been identified and characterized in preclinical studies. However, only ataluren has been approved for a subset of patients with a single disease: DMD. This difference between the vast number of preclinical studies and disappointing outcomes from clinical studies is striking (Figure 1).

To investigate factors contributing to this translational gap, we review preclinical and clinical research related to readthrough compounds. We first summarize the characteristics of the most recently discovered and validated readthrough compounds, focusing on the types of assays in

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which those compounds were discovered and validated. We then review the outcomes of clinical trials of readthrough compounds. This double-sided overview will give insight into which preclinical and clinical aspects contribute to the observed translational gap. Finally, we summarize which steps could contribute to advancing the field of readthrough biology (Figure 2, Key figure).

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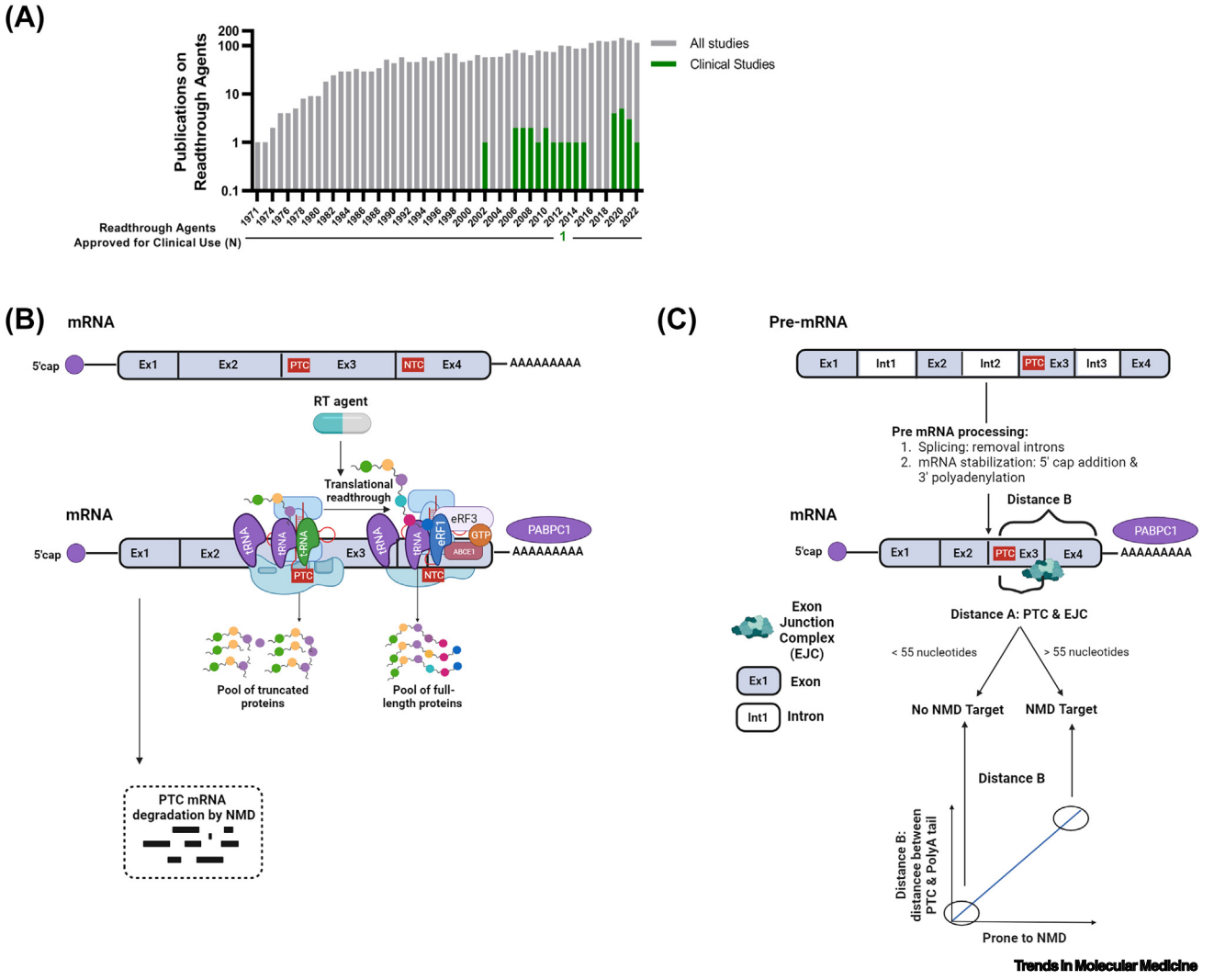

Figure 1. Translation termination in health and disease. (A) Overview of the number of publications on readthrough (RT) compounds found in PubMed (search conducted on 18 October 2022). Publications specifically on clinical trials are indicated in green, and clinical approval of RT compounds is indicated below the graph. (B) In the top panel, translation termination at a premature termination codon (PTC) site is schematically summarized. In brief, eukaryotic release factor 1 (eRF1), that structurally resembles a tRNA, binds directly to the PTC site located in the ribosomal acceptor (A)-site [7]. The GTPase eRF3 then hydrolyses GTP to GDP resulting in a conformational change of eRF1, promoting peptide release and replacement of eRF3 by ATPase ABCE1. ABCE1 promotes ATP to ADP hydrolysis, resulting in recycling of the ribosomal subunits [101]. Poly(A) tail-binding protein C1 (PABPC1) resides on the poly(A) tail and interacts with eRF3 to promote translation termination [102]. Subsequently, PTC mRNAs are rapidly degraded by nonsense-mediated decay (NMD). The bottom panel shows that compounds with RT activity can induce ribosomal RT at the PTC site, resulting in full-length protein. (C) Schematic of NMD and the two main routes resulting in NMD [103]. Because exon junctions always precede the natural termination codon (NTC), an **exon junction complex (EJC)** downstream of a stop codon indicates the presence of a PTC (distance A). In addition, the increased distance between PTC sites and proteins binding to the poly(A) tail such as PABPC1 is a second trigger for the NMD process to start (distance B). Although eRF3 plays a role in translation termination regulation, it is also involved in NMD, as do up-frameshift (UPF) proteins [9] and suppressor of morphogenesis in genitalia (SMG) proteins [104]. Abbreviations: Ex, exon; Int, intron.

Translation termination and readthrough in health and disease

During translation, tRNAs charged with AAs bind to the ribosomal acceptor-site (A-site) via its anticodon sequence. Consequently, the tRNA-associated AA is coupled to the growing polypeptide chain. Translation termination occurs when the ribosomal A-site reaches one of the three natural termination codons (NTCs): UAA, UAG, or UGA. Translation termination is heavily regulated by an intricate termination complex, of which **eukaryotic release factor** 1 (eRF1) and eRF3 are key components [7,8]. The presence of a PTC mutation upstream of the NTC results in aberrant translation termination and consequently the production of truncated protein (Figure 1B). Moreover, PTC-bearing mRNAs are degraded by the **nonsense-mediated decay (NMD)** machinery to prevent the accumulation of potentially hazardous truncated proteins [9]. NMD is a heavily regulated quality control mechanism and has recently been reviewed in great detail by Lejeune, Supek, and colleagues [10,11]. Based on the characteristics of PTC harboring mRNAs, two main routes for NMD induction have been proposed (Figure 1C).

Under native conditions, a small fraction (<0.1%) of PTCs undergo translational readthrough [12]. This is even less frequent at NTCs owing to PTC–NTC differences such as the increased distance of PTCs from the 3′-end and thus reduced interaction with translation termination agonists such as poly(A) tail-binding proteins (PABPs). In addition, multiple alternative stop codons are often found downstream of the NTC, further decreasing the chance of extensive readthrough of the 3′-untranslated region (UTR) [8,13,14]. Importantly, this absence of NTC quality control mechanisms at PTC sites provides a rationale for the development of therapies that can selectively increase readthrough at PTCs over NTCs.

Readthrough compounds: classes and efficacy determinants

Readthrough compounds act through various modes of action (MoAs) to promote the incorporation of near-cognate tRNAs that outcompete the translation termination complex and consequently drive translation beyond the PTC site (Figure 3A) [6,15,16]. The first class of identified readthrough compounds were aminoglycosides, originally used as antibiotics, that inhibit ribosome proofreading fidelity – thereby increasing the chance of non-cognate tRNAs binding to the PTC [17]. Although initial results on the readthrough activity of aminoglycosides were promising, their use was associated with persistent and serious ototoxicity and nephrotoxicity [18]. Other categories encompass compounds such as CC90009 and SRI-41315 that inhibit factors of the translation termination complex [19,20], compounds such as **2,6-diaminopurine (DAP)** that reduce the fidelity of specific tRNAs [21], and nucleoside analogs such as clitocine [22]. For several readthrough compounds, the exact MoA has not been clarified.

Multiple factors influence the efficacy of most readthrough compounds irrespective of their MoA [23]. In brief, the main determinants are (i) mRNA transcript levels, depending on the gene in question and overall NMD activity, (ii) PTC identity, location, and sequence context, and (iii) the characteristics of the affected protein (Table 1).

The search for novel and optimized readthrough compounds can be divided into three main categories: (i) high-throughput compound screening, (ii) computational approaches, and (iii) empirical, hypothesis-driven approaches (Figure 3B). In the next section we review preclinical studies on the most recently discovered and validated readthrough compounds, focusing on the types of assays in which those compounds were discovered and validated.

Readthrough compound discovery

The first widely used assays to measure readthrough were based on expression of PTC-bearing luciferase transgenes in cell lines, enabling sensitive readthrough measurements through

Glossary

Alport syndrome: a genetic disorder caused by a defect in type IV collagen.
Anticodon-engineered tRNAs (ACE-tRNAs): tRNAs that induce readthrough by binding to the premature termination codon (PTC) site and incorporating the amino acid they encode.
Cystic fibrosis (CF): an autosomal, recessive disorder that is one of the most studied genetic diseases in the context of nonsense mutations. CF is caused by mutations in the *CFTR* gene that encodes an apical cell surface chloride channel that maintains ion homeostasis, viscosity, and hydration of secretions, and that malfunctions in patients with CF.
2,6-Diaminopurine (DAP): a non-aminoglycoside readthrough compound that acts by inhibiting methyltransferase 1 (FTSJ1), thereby decreasing methylation of $tRNA^{Trp}$, which in turn decreases its fidelity and results in recognition of UGA PTC mutations as tryptophan (UGG)-encoding codons.
Duchenne muscular dystrophy (DMD): an X-linked progressive disease caused by a mutation in the dystrophin gene. Dystrophin encodes a protein that stabilizes cell membranes and influences muscular strength. DMD is the only disease for which a readthrough compound (ataluren) is clinically approved.
Exon junction complex (EJC): consists of proteins that are localized at the ends of two assembled exons. A PTC results in the presence of EJCs after the PTC site, inducing mRNA degradation by NMD.
Eukaryotic release factors: are involved in promoting translation termination. As such, their partial inhibition could be exploited as a readthrough strategy.
Forskolin-induced swelling (FIS): the FIS assay is a preclinical, functional assay on patient-derived intestinal organoids that allows measurement of CFTR function. It is one of a few preclinical assays for which it has been shown that outcomes correlate with clinical parameters.
Nonsense-mediated mRNA decay (NMD): a surveillance mechanism that rapidly degrades PTC-containing mRNAs to prevent the accumulation of high levels of truncated proteins.
Readthrough: incorporation of an amino acid at a termination codon, either

detection of chemiluminescence. Owing to the straightforward read-out, luciferase reporter systems have been used in many high-throughput screens for readthrough compound discovery. For instance, they were used to demonstrate the readthrough activity of gentamicin, G418, paromomycin, clitocine, and amlexanox [6,24,25]. Ataluren was discovered in a similar manner in an ultra-high-throughput screen of ~800 000 compounds [26]. In follow-up experiments, however, ataluren was found to directly bind to and stabilize firefly luciferase, indicating an indirect, false-positive effect in previous studies [27]. Subsequent validation studies yielded conflicting

a natural termination codon or a PTC due to a nonsense mutation. Readthrough can occur naturally, or can be induced via readthrough compounds or other strategies.

Key figure

Readthrough compounds for nonsense mutations: bridging the translational gap

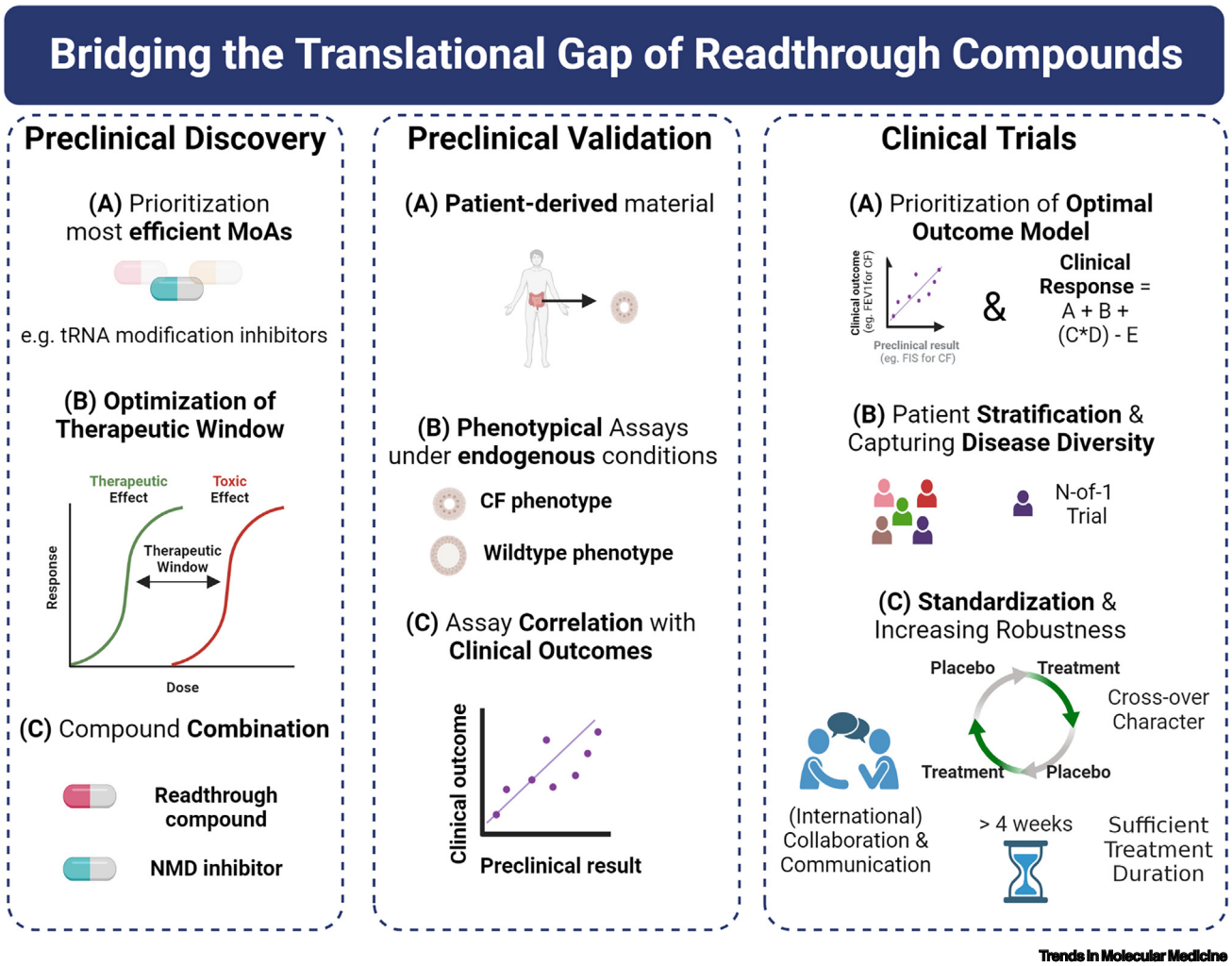

Figure 2. There is a large translational gap between the success of readthrough compounds in preclinical studies and the absence of effect in clinical trials. To bridge this gap, the field should give attention to several aspects: (i) the preclinical discovery phase (left panel) could prioritize the development of readthrough compounds with more potent MoAs, (ii) the preclinical validation phase (middle panel) could employ preclinical assays that correlate with clinical outcomes, and (iii) clinical trials (right panel) could, for example, prioritize the development of more complex and complete clinical outcome models. Abbreviations: CF, cystic fibrosis; MoA, mode of action; NMD, nonsense-mediated decay.

(A)

	Category	RT agents
1	Inhibition of proofreading mechanism of the ribosome	G418 [6], ●Gentamicin [6], Negamycin [47], ●ELX-02 [16]
2	UPF machinery inhibition	▲■●◆Ataluren [29], RTC13/RTC14 [48], GJ071/GJ072 [49], CC-90009/CC-885 [19], SRI-37240/SRI-41315 [20]
3	mRNA/tRNA mispairing	▲■●◆Ataluren [29], RTC13/RTC14 [48], GJ071/GJ072 [49], NV848/NVN914/NV930 [59], DAP [21]
4	Incorporation of nucleoside analogs	Clitocine [22]
5	Unknown	Amlexanox [28], Escin [45], Y320 (52), ●Azithromycin [51], ●Erythromycin [51], ●NPC-14

Phase 2
Phase 3
Phase 4
Market-approval

(B)

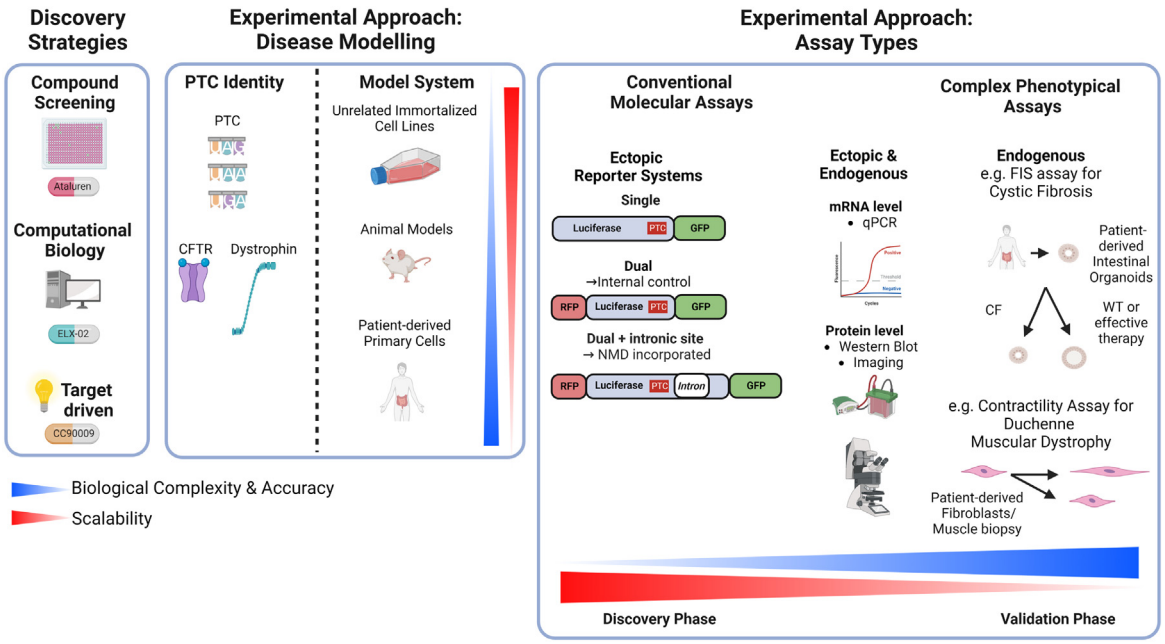

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Table 1. Summary of determinants that influence the efficacy of readthrough compounds[a]

Determinant	Depends on	Potential to interfere	Refs[b]
mRNA transcript levels	(i) Nonsense-mediated decay (NMD) activity (ii) Stability of the mRNA	(i) Yes, inhibition of NMD (ii) Yes, mRNA stabilization by amplifiers	(i) [64,87] (ii) [92]
PTC identity, location, and the surrounding sequence	(i) The proximity of the PTC to the NTC affects the size and function of the truncated peptide (ii) Readthrough susceptibility (UGA < UAG < UAA) and the sequence surrounding to PTC (iii) Identity of the amino acid incorporated	(i) No (ii) No (iii) Yes, depending on the mode of action of the readthrough compound	(i) NA (ii) [14] (iii) [21]
Protein identity	(i) Protein half-life (ii) Minimal amount of protein for proper function	(i) No (ii) Partially, protein modulators can aid in proper folding and protein function The overall minimal amount of protein for sufficient function is disease-dependent	(i) NA (ii) [64,87]

[a]Determinants are divided into three main categories, affecting (i) mRNA transcript levels, depending on the gene in question and overall NMD activity, (ii) PTC identity, location, and its sequence context; and (iii) the characteristics of the affected protein.
[b]NA, not available.

results [28] because no readthrough activity was demonstrated in cell models for obesity [29], peroxisome biogenesis disorders [30], long-QT syndrome [31], or CF [32]. However, readthrough activity of ataluren was demonstrated in other experiments for the same and other disease models, including Usher syndrome [33], Miyoshi myopathy [34], DMD [26], and CF [35].

Although luciferase reporter assays are sensitive detectors of readthrough, they can result in compounds with readthrough activity that is negligible in a physiological setting. Updated versions of luciferase reporters include dual reporters that enable internal expression normalization and thereby increase the robustness of readthrough quantification [36–39], as well as intronic reporters that allow screening in the context of NMD [21,40]. Dual reporter assays were instrumental in the discovery of NB30 and NB54 [41] and were used to reassess the readthrough activity of gentamicin, amikacin, negamycin, and escin [42–44]. Intronic reporter assays resulted in the discovery of the readthrough capacity of DAP in a 164 compound medium-throughput screen [40]. SRI-37240 and SRI-41315 were similarly discovered in an ultra-high-throughput screen of ~771 345 compounds [19].

Alternative approaches have been designed to complement luciferase-based assays. Du *et al.* developed a protein-based transcription-translation (PTT) ELISA to screen 34 000 compounds and identified 12 non-aminoglycosides with readthrough activity, including RTC13 and RTC14 [45]. Screening of a second library of ~36 000 compounds resulted in

Figure 3. Ribosomal readthrough as therapeutic strategy. (A) The modes of action (MoAs) of readthrough compounds can be divided into five main categories. Category 1 compounds, which include aminoglycosides, interact with 16S rRNA at the ribosomal acceptor (A)-site to inhibit stop codon recognition by release factors. Consequently, near-cognate tRNAs can outcompete the translation termination complex [6,16,44]. Category 2 compounds directly inhibit components of the translation termination complex. For example, CC-90009 and CC-885 have been shown to inhibit eRF1 and eRF3 levels [19,20,26,45,46]. Category 3 compounds reduce the fidelity of near-cognate tRNAs, thus increasing their chance of binding to stop codons [21,26,45,46,56]. Category 4 compounds are nucleoside analogs that are incorporated into the mRNA during transcription and become part of the premature termination codon (PTC) [22]. Such PTCs are not recognized by eRF1 as a stop codon, increasing the probability of near-cognate tRNA insertion. Category 5 compounds include compounds for which the exact MoA has not been clarified [25,42,48,49]. (B) A schematic of the experimental approaches towards readthrough compound discovery and validation. In the discovery phase (left panel), three different approaches can be employed: high-throughput screening, computational biology, and target-driven research. In the compound validation phase, experimental approaches can be discriminated based on (i) the gene studied and PTC identity, and (ii) the model system employed, ranging from conventional 2D cell lines and more complex patient-derived organoids and animal studies (middle panel). The main assay types used for either compound discovery or validation are illustrated in the right panel and can be divided into (i) ectopic assays in which PTC-harboring DNA constructs are used that allow high-throughput reporter system assays, (ii) endogenous conventional protein detection assays such as western blotting, and (iii) complex phenotypic assays such as the forskolin-induced swelling (FIS) assay for cystic fibrosis (CF). Abbreviation: WT, wild type.

discovery of GJ071 and GJ072 [46]. Alternatively, Liang *et al.* measured readthrough by fusing a PTC-bearing cystic fibrosis transmembrane conductance regulator (*CFTR*) transgene to the horseradish peroxidase (HRP) gene whose protein product is detectable through its chemiluminescence activity [47]. Although both PTT-ELISA and *CFTR*-transgene-HRP assays may complement luciferase-based reporter systems, they could similarly produce false-positive hits that augment HRP activity. A different dual fluorescent reporter system with a readout based on flow cytometry was developed by Caspi and colleagues, resulting in discovery of azithromycin and erythromycin as readthrough compounds [48]. Their vector system – which is not based on luciferase expression – has advantages over luciferase-based constructs which have shown to interact directly with, for example, ataluren. Detecting protein levels from the endogenous gene of interest rather than transgenic assays is generally less sensitive and less scalable, but its hit detection is likely more robust. Y320 was discovered in this manner, and increased readthrough of endogenous $TP53^{637C>T}$ (encoding TP53-R213X) as measured with immunofluorescence microscopy [49].

Unbiased high-throughput screening methods contrast with hypothesis-driven approaches. Baradaran-Heravi *et al.* sought to inhibit release factors, hypothesizing that their depletion increases the chance of ribosomal readthrough by stalling translation termination at stop codons [20]. Compounds CC-885 and CC-90009, that are known to degrade eRF3a, indeed enhanced readthrough in a panel of disease models.

Overall, the discovery phase in general requires high-throughput assay capacity which is often best achieved with exogenous reporter systems, as exemplified in studies on DAP, ataluren, SRI-41315, RTC13/RTC14, and GJ071/GJ072. However, it is essential that the outcomes of such experiments are validated in more complex, phenotypic experiments in patient-derived model systems that capture the complex biology of the disease in question.

Readthrough compound validation

A first challenge after compound discovery is the subsequent hit-to-lead drug development process in which hits are optimized in terms of safety, efficacy, and pharmacokinetic properties. Computational chemistry has played an important role in this hit-to-lead stage, for example, by decreasing the toxicity and increasing the efficacy of aminoglycoside derivatives ataluren [50–54] and ELX-02, which were shown to be less toxic than gentamicin or G418 [16,37,55,56]. Ataluren derivatives NV848/NV914 and NV930 are in particular worth mentioning because their preclinical work in the context of CF is promising and recent pharmacokinetic data highlight a favorable pharmacokinetic profile in animal models [57]. In addition, a recent finding indicates that NV848/NV914 and NV930 exert an MoA similar to that of DAP, a potent readthrough compound that specifically results in incorporation of tryptophan at a UGA site [58].

Proper validation of final compounds of interest is pivotal and can be divided into two steps: (i) demonstration of target protein elevation, and (ii) demonstration of recovery of protein function. Although protein level characterization is relatively straightforward by conventional techniques such as western blotting, and has been performed in validation studies for most readthrough compounds, the confirmation of protein rescue at a functional level has proved to be more challenging.

A prime example of phenotypic, functional characterization is the measurement of CFTR function in CF patient-derived intestinal organoids (PDIOs) with the **forskolin-induced swelling (FIS)** assay [59]. Importantly, results on PDIOs obtained by FIS assays correlate with clinical results for the patient the PDIOs are derived from [60,61]. As such, observing no effect of a compound

suggests that the compound will not be effective in the clinic, which was indeed the case for ataluren [32,62]. Validation of ELX-02 in FIS assays yielded conflicting results. Although one study observed CFTR rescue with ELX-02 as monotherapy for G542X homozygous and heterozygous patient-derived cells [63], our own study showed little effect of a commercial ELX-02 variant as a single compound across a panel of CFTR PTC genotypes [64]. One readthrough compound that did induce functional recovery as monotherapy in FIS assays is DAP (our unpublished data), warranting prioritization of this readthrough compound in further (pre)clinical studies. An important remark concerning comparison of DAP to NV848/NV914/NV930 is that NV848/NV930 and NV914 fall into category 4/5 of the Globally Harmonized System (GHS) of Classification and Labeling of Chemicals, underlining their low health risk identity. DAP, however, although it was not toxic in preclinical work including mouse studies, could hold additional challenges in this

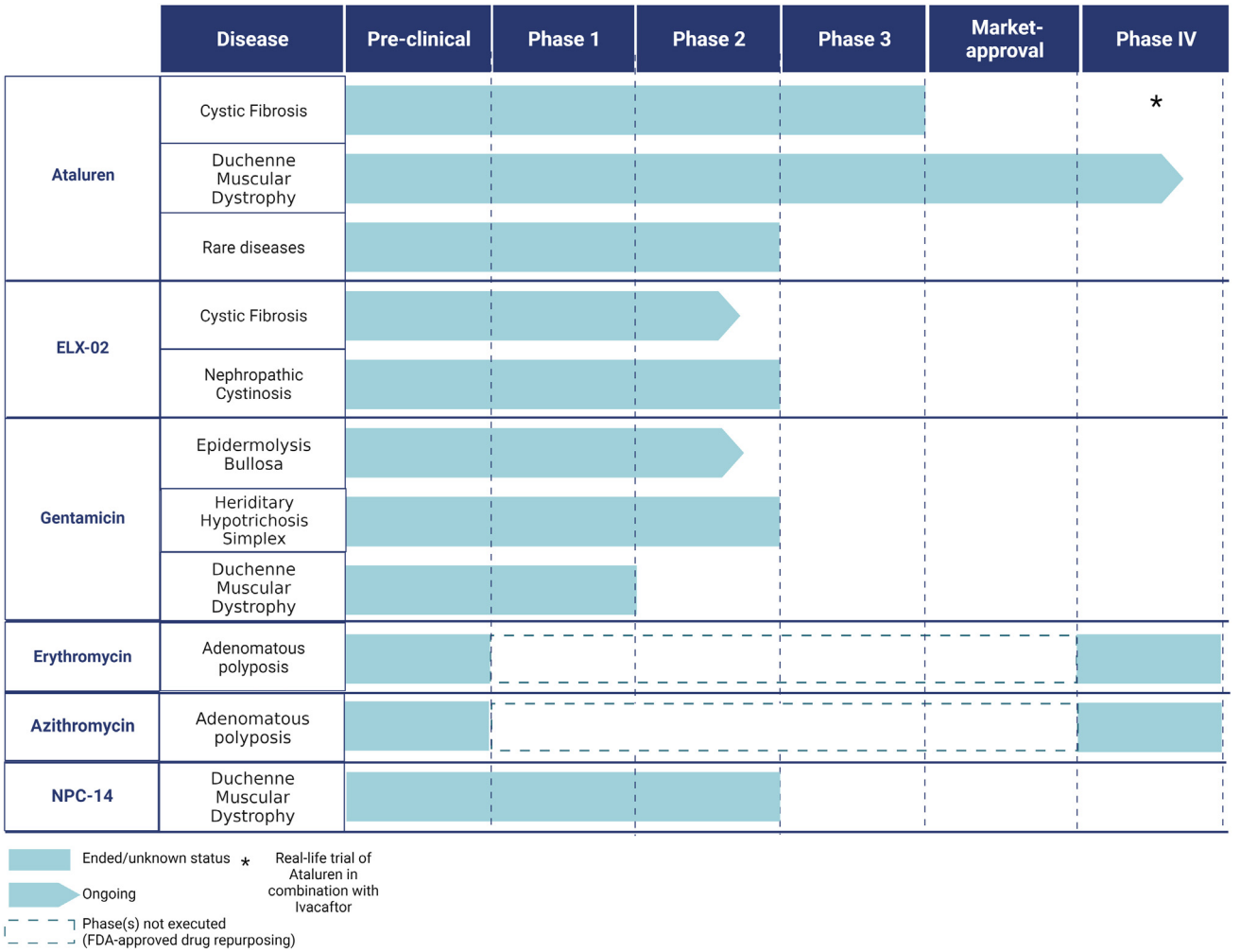

Figure 4. Readthrough compounds in clinical trials stall in Phase 2. Outline of the stage of readthrough compounds in clinical trials conducted between February 2000 and June 2022. Although the clinical development of ataluren for nonsense mutation-induced cystic fibrosis (CF) stopped after a negative outcome Phase 3 trial, two real-life setting $N = 1$ Phase 4 trials have been performed to assess ataluren efficacy in combination with ivacaftor (NCT03256968 and NCT03256799). In some cases, compounds were already FDA-approved for other disease indications, and drug repurposing can allow a faster clinical study pipeline (see Figure S1 in the supplemental information online).

Table 2. Summary of outcomes of Phase 2/3/4 clinical trials for readthrough compounds[a,b]

NCT number	Phase and number of participants (participant age)	Timeframe (weeks)[c]	Study design	ΔFEV1pp	Remarks	Refs
Ataluren for cystic fibrosis						
NCT00237380	Phase 2; *N* = 23 (≥18 years)	**2**–2–**2**–2	INT, SG, OLA	Cycle 1: ↑ Cycle 2: NA		[76]
NCT00351078	Phase 2; *N* = 19 (≥18 years)	**12**–4	INT, SG, OLA	NS		[105]
NCT00234663	Phase 2; *N* = 24 (≥18 years)	**8**–408	INT, SG, OLA, NR	NA	Trial outcomes are not available online or in publication form	
NCT00458341	Phase 2; *N* = 30 (6–18 years)	**2**–2–**2**–2	INT, CO, OLA, R	NS		[106]
NCT00803205	Phase 3; *N* = 209 (≥6 years)	**48**–4	INT, P, R	NS		[62]
				↑ + 5% (*P* = 0.0082)	*Post hoc* analysis on patients not treated with tobramycin	
NCT02139306	Phase 3; *N* = 279 (≥6 years)	**48**–4	INT, P, R	NS		[77]
NCT01140451	Phase 3; *N* = 191 (≥6 years)	**96**–4	INT, P, R	NA	Extension of NCT00803205	
NCT02107859	Phase 3; *N* = 61 (≥6 years)	**192**–4	INT, SG, OLA		Terminated due to lack of success of NCT02139306	
NCT02456103	Phase 3; *N* = 246 (≥6 years)	**96**–4	INT, SG, OLA			
NCT03256968	Phase 5; *N* = 1 (31 years)	**48**–368	INT, SG, OLA	NS	Combined with ivacaftor	[78]
NCT03256799	Phase 5; *N* = 1 (32 years)	**48**–368	INT, SG, OLA	NS	Combined with ivacaftor	[78]
Ataluren for Duchenne muscular dystrophy						
NCT00264888	Phase 2; *N* = 38 (≥5 years)	**4**–244	INT, NR, SG, OLA	NA	Trial outcomes are not available online or in publication form	[81]
NCT00592553	Phase 2; *N* = 174 (≥5 years)	**48**–6	INT, R, P, QM	NS (*P* = 0.056)	Longer before 10% decline in 6MWD	[82]
NCT00759876	Phase 2; *N* = 36 (all ages)	**96**–0	INT, SG, OLA		Terminated due to lack of efficacy of ataluren in NCT00592553	
NCT00847379	Phase 2; *N* = 173 (≥5 years)	**96**–6	INT, SG, OLA			
NCT01009294	Phase 3; *N* = 6 males (≥7 years)	**50**–366	INT, SG, OLA			
NCT02090959	Phase 3; *N* = 219 (7–15 years)	**144**–6	INT, R, P, QM		Terminated, due to withdrawal of sponsor and commercial availability of ataluren for DMD	
NCT01826487	Phase 3; *N* = 230 (7–16 years)	**48**–6	INT, R, P, QM	NS	All outcomes positive in a prespecified group, 6MWD >300 and <400 m	[80]
NCT01557400	Phase 3; *N* = 94 (all ages)	**240**–6	INT, SG, OLA	NS		[79]
NCT02819557	Phase 2; *N* = 14 (2–5 years)	**52**–4	INT, SG, OLA	NA	Trial outcomes are not available online or in publication form	
NCT03796637	Phase 2; *N* = 6 (all ages)	NA	INT, SG, OLA	NA		
NCT03648827	Phase 2; *N* = 20 (2–7 years)	NA	INT, SG, OLA	NA		
NCT04336826	Phase 2; *N* = 10 (6 months–2 years)	**24**–52	INT, SG, OLA		Recruiting	
NCT01247207	Phase 3; *N* = 270 (all ages)	**384**–4	INT, SG, OLA		Enrolling by invitation	
NCT03179631	Phase 3; *N* = 360 (≥5 years)	NA	INT, R, P, QM		Active, not recruiting	[107]
Ataluren for methylmalonic acidemia						
NCT01141075	Phase 2; *N* = 11 (≥2 years)	**96**–48	INT, SG, OLA	NA	Terminated due to low patient enrollment	
Ataluren for hemophilia						
NCT00947193	Phase 2; *N* = 13 (≥18 years)	**2**–2–**2**–2	INT, SG, OLA	NA	Terminated, sponsor withdrawal	
Ataluren for Dravet syndrome (DS) and CDKL5 deficiency syndrome (CDD)						
NCT02758626	Phase 2; *N* = 16 (2–12 years)	**12**–4–**12**–4–16	INT, R, CO, QM	NS		[108]

(continued on next page)

Table 2. (continued)

Ataluren for cystic fibrosis						
NCT number	Phase and number of participants (participant age)	Timeframe (weeks)[c]	Study design	ΔFEV1pp	Remarks	Refs
Ataluren for aniridia						
NCT02647359	Phase 2; *N* = 39 (≥2 years)	**240**–4	INT, R, P, QM	NS		
NCT04117880	Phase 2; *N* = 0 (≥2 years)	NA	INT, SG, OLA		Withdrawn	
ELX-02 for cystic fibrosis						
NCT04135495	Phase 2; *N* = 16 (≥18 years)	**9**–0	INT, R, P, QM		Recruiting	[77]
NCT04126473	Phase 2; *N* = 16 (≥16 years)	**9**–0	INT, SG, OLA		Recruiting, extension of NCT04135495, ivacaftor addition	[78]
ELX-02 for nephropathic cystinosis						
NCT04069260	Phase 2; *N* = 3 (≥12 years)	6–**4**–4	INT, SG, OLA	NA	Terminated prematurely due to study design limitations	
Gentamicin for junctional epidermolysis bullosa (JEB)						
NCT04140786	Phase 2; *N* = 6 (all ages)	**12**–12.8 **3.4**–12.8	INT, NR, OLA	NA	Primary outcome is safety, not an efficacy-associated parameter	[109]
NCT03526159	Phase 2; *N* = 5 (all ages)	**2**–10	INT, SG, OLA		Recruiting	
Gentamicin for recessive dystrophic epidermolysis bullosa (RDEB)						
NCT03012191	Phase 2; *N* = 6 (all ages)	**24**[d]	INT, SG, OLA	NA	Primary outcome is safety, not an efficacy-associated parameter	
NCT03392909	Phase 2; *N* = 9 (≥7 years)	**2**–22 **12**–12	INT, SG, OLA	NA	Trial outcomes are not available online or in publication form	
NCT02698735	Phase 2; *N* = 5 (all ages)	**12**[d]	INT, NR, P, TM	NA	Increase in anchoring fibrils (AFs) and wound closure, significance NA	[85]
Gentamicin for hypotrichosis simplex						
NCT03492866	Phase 2; *N* = 8 (18–12 years)	**2**–10	INT, NR, CO, OLA	NA	Trial outcomes are not available online or in publication form	
Erythromycin for adenomatous polyposis						
NCT02175914	Phase 5; *N* = 20 (≥18 years)	**12**–36	INT, SG, OLA	NA	Trial outcomes are not available online or in publication form	
NCT02354560	Phase 5; *N* = 30 (6–18 years)	**16**–32	INT, SG, OLA	NA	Trial outcomes are not available online or in publication form	
Azithromycin for adenomatous polyposis						
NCT04454151	Phase 5; *N* = 15 (10–17 years)	**16**–48	INT, SG, OLA	NA	Trial outcomes are not available online or in publication form	
NPC-14 for Duchenne muscular dystrophy						
NCT01918384	Phase 2; *N* = 21 males (≥4 years)	**36**–2	INT, R, P, TM	NA	Trial outcomes are not available online or in publication form	

[a]Abbreviations: ↑, improved outcome; CO, crossover; ΔFEV1pp, forced expiratory volume in one second; INT, interventional; 6MWD, 6 minute walk distance; NA, not available; NR, not randomized; NS, not significant; OBS, observational; OLA, open label assignment; P, parallel; PROS, prospective; QM, quadruple masking; R, randomized; SG, single group; TM, triple masking.
[b]Overview of primary and secondary outcomes in clinical trials on readthrough compounds. Results were pooled in case of separation into groups (e.g., for different doses). If statistical testing of outcome measurements is not described in a publication or on ClinicalTrials.Gov, outcome results are summarized as 'NA'.
[c]Timeframe: treatment time (in weeks) is given in bold font, follow-up time in regular font.
[d]The timeframe (e.g., follow-up vs. treatment time) in these cases is not clearly described.

respect owing to (i) its original characterization as an anticancer reagent, and (ii) the simplicity of its structure as an adenosine analog, but there is ample space for biochemical and structural optimization, for example, to optimize the therapeutic window.

In addition to the FIS assay, electrical current measurements on patient-derived airway cells correlate with clinical outcome measurements in CF patients [65,66]. The development of predictive preclinical assays has proved to be more challenging for other genetic diseases, including DMD. However, recently developed muscle cell contractility assays on iPSC-derived muscle cells show promise as functional assays for DMD patient-derived material [67–69]. Prioritization of those assays whose outcomes correlate with clinical disease manifestation is pivotal.

Finally, animal models can provide additional systemic and metabolic complexity in terms of validating compounds and associated metabolites for efficacy and toxicity. The differences between various *in vivo* models have been extensively reviewed (e.g., CF [70] and DMD [71]). In the context of nonsense mutations, rodent models are the most widely used. However, some animal models are known to incompletely or inaccurately model human diseases, as evidenced by intestinal obstruction being the most common morbidity in CF mouse models whereas airway obstruction is less prominent. An often used readout in the context of CF is the FIS assay on mouse-derived intestinal organoids. In such settings it has been shown, for example, that G418 restores CFTR function in homozygous G542X mouse organoids whereas ataluren did not [72]. In a previous study, however, ataluren did result in reappearance of CFTR protein in intestinal glands as well as partial restoration of function based on current-based measurements in *Cftr*$^{-/-}$ G542X mice [73]. It can therefore be debated whether animal models have sufficient translational value in all elements of hit validation. For DMD, muscle function can be functionally assessed in mouse models, thus providing additional advantages over cellular models that simplify the complexity of the muscle system. As such, ataluren promoted dystrophin expression in primary muscle cells in mice expressing dystrophin nonsense alleles, and also rescued muscle function [26]. These differences between (i) the efficacy of readthrough compounds in different disease contexts, and (ii) positive results obtained in mouse studies with ataluren versus absence of clinical efficacy of ataluren, are striking and underline the complexity of animal studies. Prioritization of non-rodent animal models for CF and DMD could be beneficial in the context of nonsense mutations. For CF, the pig model could hold additional value because (i) porcine *CFTR* is genetically very similar to human *CFTR*, and (ii) the porcine respiratory system has many similarities to the human respiratory system in terms of anatomy, size, and physiology [74]. However, to date porcine models with CF nonsense genotypes have not been developed. With regard to DMD animal models, dystrophin-deficient dogs recapitulate the DMD clinical course in many aspects [71]. Canine models with dystrophin PTC mutations are not yet available, but may provide a great asset for validation of readthrough compounds in the context of DMD.

Readthrough compounds in clinical trials

A systematic search for clinical studies on readthrough compounds resulted in a total of 48 clinical trials (Figure S1 in the supplemental information online). Because this review focuses on the clinical efficacy of readthrough compounds, we do not elaborate on Phase 1 trials and safety endpoints. Overall, no severe side effects in any of the trials were reported. Ataluren, ELX-02, and gentamicin have been the most extensively studied for various disease indications (Figure 4). Outcome measures of finalized trials for those readthrough compounds are described in Tables S1–S4 in the supplemental information online. We summarize these finalized trials as well as ongoing trials in Table 2, and highlight the outcome for the most relevant clinical parameter in each disease.

Interpretation of readthrough compound efficacy across clinical trials is challenging owing to differences in research design, treatment length, and clinical endpoints. In the context of rare

Clinician's corner

Readthrough drugs comprise an area of intense preclinical investigation owing to the potential applicability to genetic disorders caused by nonsense mutations, and for which no treatment is available today.

In contrast to results obtained in preclinical studies, readthrough drugs have not yielded success in clinical trials, with the exception of ataluren for a subset of patients with Duchenne muscular dystrophy. To increase the translatability of preclinical results, assays that correlate with clinical outcome measures should be prioritized. Such assays should (i) recapitulate disease biology, for example, using patient-derived cells, and (ii) have a functional/phenotypic character that characterizes the function of the targeted gene, instead of conventional experiments in which protein amounts are characterized.

It is pivotal that such preclinical assays are compared to clinical data to establish correlations between preclinical results and clinical outcome. Deciding on the optimal clinical outcome for establishing those correlations is essential. In the case of a small effect size and/or small population size, it can be beneficial to develop a composite endpoint model in which several outcome measurements are combined into a single statistical model.

Because numbers of affected patients are low and the effect size is small, clinical trials set-ups need to be optimized. We recommend (i) standardization of clinical trials, mainly focusing on outcome measures, treatment length, and dosage; (ii) increasing statistical power by performing repeated measures and on/off cycles, and performing $N = 1$ trials allowing a personalized approach.

Although improvements in preclinical and clinical study design may lead to a better assessment of readthrough compounds, the overall issue of limited drug efficacy requires further attention. In particular, degradation of premature termination codon (PTC)-harboring mRNAs by the nonsense-mediated decay (NMD) quality control system results in an additional

diseases and small population sizes, crossover (CO) designs, in which participants receive treatment and placebo sequentially in a randomly selected order, can be useful [75]. An important requirement for CO studies is that responses are rapidly detectable, which can be challenging depending on the disease and disease stage. In the context of readthrough compound trials, only three clinical studies had a CO design (NCT00458341, NCT02758626, and NCT03492866). Depending on disease and disease stage, it is pivotal that treatment length is sufficient to result in changes in clinical parameters. It can be questioned whether trials with treatment periods for 1 month or shorter (e.g. NCT00458341) allowed the detection of such changes.

challenge when it comes to effective readthrough therapy. To date, no NMD inhibitor has been characterized in a clinical setting, and further development of NMD inhibitors with an emphasis on reducing toxicity is warranted.

In the context of CF, the first ataluren trial indicated a significant improvement in forced expiratory volume (FEV1) (NCT00237380), but this was not confirmed in a large Phase 3 trial (NCT00803205) [62,76]. *Post hoc* analyses showed that use of antibiotic tobramycin was associated with lack of efficacy, which was confirmed in preclinical analyses (NCT00803205) [62]. Unfortunately, a Phase 3 follow-up study in patients not receiving aminoglycosides (NCT02139306) did not show clinical improvement [77]. A combination of compounds that target different cellular mechanisms could enhance treatment efficacy; however, both Phase 4 trials in which ataluren and the CFTR modulator ivacaftor were combined in a single patient ($N = 1$ trial) did not report clinical improvement (NCT03256968 and NCT03256799) [78], ending clinical trial investigations of ataluren in the context of CF [78]. In the context of DMD, ataluren has been found to stabilize DMD patients in the mid-range phase of the disease [79,80]. However, multiple clinical trials with ataluren in patients with DMD yielded conflicting outcomes, likely owing to overall lack of efficacy but possibly also reflecting differences in trial set-up, disease stage and outcome measures [81,82]. Nevertheless, ataluren received conditional approval in July 2018 from the European Medicines Agency (EMA) for treating pediatric, ambulatory DMD patients (≥2 years). Post-marketing studies are currently recruiting patients to further evaluate ataluren in DMD (NCT03179631, NCT01247207).

ELX-02 is currently being evaluated in two Phase 2 trials (NCT04126473 and NCT04135495) in CF patients with at least one PTC allele. Preliminary results showed a minor decrease in sweat chloride concentration, indicating only limited treatment efficacy [83]. ELX-02 is additionally being investigated in combination with the CFTR modulator ivacaftor (NCT04135495); however, a first press release reported no significant improvement upon this dual therapy [84]. Another Phase 2 trial of ELX-02 in nephropathic cystinosis patients (NCT04069260) was initiated but has been halted prematurely owing to design limitations. A Phase 2 trial with ELX-02 and patients with **Alport syndrome** is expected to start in 2023.

Gentamicin has been investigated in Phase 1/2 trials in several rare diseases including the skin disorder recessive dystrophic epidermolysis bullosa (RDEB). RDEB patients who received gentamicin exhibited a persistent increase in collagen VII expression and enhanced wound healing (NCT02698735) [85].

Erythromycin, azithromycin, and NPC-14 have been investigated for various disease indications, but no results have been published, suggesting absence of effect in a clinical setting (NCT02175914, NCT02354560, NCT04454151, NCT01918384).

Overall, although various clinical studies of readthrough compounds have been initiated, this has not resulted in encouraging outcomes except for the conditional approval of ataluren for a subset of DMD patients.

Concluding remarks

Readthrough biology remains an area of intense investigation because of its potentially large impact for the ~10% of genetic disorders that are caused by nonsense mutations. Despite the development and preclinical validation of >30 different readthrough compounds, only ataluren is clinically approved for a subset of DMD patients. To increase the chances of readthrough compounds reaching the clinic, it is essential to characterize which elements in preclinical discovery, preclinical validation, and clinical study stages could be improved (Figure 2). Preclinically, prioritization of the development of functional preclinical models that correlate with clinical outcomes is likely to result in better prediction of drug efficacy in the clinic. Clinically, a main challenge lies in patient stratification for the first clinical trials and in clinical trial standardization to allow comparison of different studies.

Overall, preclinical studies yield promising results when simplified experimental approaches are undertaken. However, when more complex validation experiments are performed (such as FIS assays in the context of CF [32,64]), results for readthrough compounds as monotherapy have been disappointing overall. Although the scalability of readthrough reporter assays hold value in the discovery phase, they lack the genetic and physiological context of the targeted genetic disorder. In particular, single or dual reporter assays are sensitive readthrough detectors and have resulted in a vast amount of positive preclinical results. Although both types are valuable for finding new leads on compounds with readthrough activity, the use of intronic reporter assays could be beneficial by selecting molecules with higher readthrough capacity in the context of NMD.

Irrespective of the type of discovery assay, it is pivotal that hits are validated with disease-specific functional assays. Currently, functional assays on patient-derived cells where outcomes correlate with clinical outcome are available for CF in particular. Prioritizing further development of such assays for other genetic diseases is warranted as well as achievable; for example, in DMD promising results have been achieved with muscle cell contractility assays on patient iPSC-derived muscle cells. A pivotal element in making the most of these patient-derived, functional preclinical assays is that the outcomes of such preclinical assays should be compared with clinical outcome measures to guide prioritization. For additional validation experiments that complement patient-derived models, the development of porcine and canine models with nonsense mutations could be beneficial in providing additional systemic complexity in comparison to conventional rodent models.

Limited clinical efficacy and small population sizes in trials can hamper the establishment of such correlations. For this reason it can be beneficial to develop a composite endpoint model in which several outcomes measurements are combined into a single statistical model. In particular, Bayesian methods allow probability calculations to test whether a treatment is likely to lie in the effective range based on all available trial and external data including patient long-term history, preclinical experiments, and the literature [86]. Importantly, a correlation at the group level does not necessarily interpolate to the individual patient. Therefore, $N = 1$ trials with *a priori* preclinical stratification could further increase our knowledge on readthrough compound efficacy.

Overall, unfortunately, the clinical trial results discussed herein have been disappointing. Although the main reason for this absence of effect is presumably lack of efficacy, the vast differences in set-ups and outcome measurements between clinical trials hamper the drawing of overarching, firm conclusions. Clinical trial standardization is therefore essential. In addition, implementation of specific elements in clinical trial design may improve the detection of clinical benefit, such as (i) repeated measurements of clinical outcomes, (ii) trials with a CO design, (iii) treatment duration

Outstanding questions

Robust preclinical assays on patient-derived material are available for CF, but not for other genetic diseases caused by PTCs. Can we prioritize the development of such assays to increase the translatability of preclinical studies?

It can be challenging to choose which clinical parameter is optimal to develop correlations between preclinical studies and clinical outcome. How should we approach this challenge?

Future studies should focus on readthrough compounds with novel and more potent MoAs, such as the readthrough compound DAP which affects $tRNA^{Trp}$ fidelity. Can other readthrough compounds with similar MoAs be discovered or developed for other PTC genotypes?

It is possible that currently existing readthrough compounds will not be sufficiently potent as monotherapy because of degradation of PTC-harboring mRNAs by the NMD machinery. Because no NMD inhibitors are currently under investigation in clinical trials, studies should focus on developing NMD inhibitors that have a favorable therapeutic window.

Standardization of clinical trials to draw more firm, overarching conclusions is essential. Specifically, standardization of trial set-up, treatment duration, and clinical end-points per disease should be prioritized.

New technologies such as CRISPR and ACE-tRNAs will enable the development of new readthrough compounds. What can be done to prevent these novel technologies from encountering the same translational gap as current readthrough compounds?

of >4 weeks (depending on disease and disease stage), and (iv) an international collaborative effort to ensure that disease and PTC diversity is captured.

Finally, it could be beneficial to perform Phase 2 trials in a subselection of patients who may be most amenable to efficient readthrough compound therapy. The exact requirements of such a preselection will be disease- and compound-dependent, but such strategies could include (i) selection of homozygous PTC patients who have two alleles for readthrough compounds to act on, preferably UGA mutations because these have been shown to be most prone to readthrough therapy, or patients selected based on the MoA of the investigated readthrough compounds (e.g., selection for PTCs where originally a tryptophan was encoded, because DAP specifically results in restoration of a tryptophan at the PTC site) [14], (ii) selection of patients who are in a stage of the disease in which clinical benefit is still expected from therapy, and (iii) preselection based on informative preclinical assays on patient-derived material. Hereafter, in case of positive outcomes, additional Phase 2/3 trials with different stratification strategies can further allow stratification of patients or patient groups who could benefit from the therapy.

Although improvements in preclinical and clinical study design may lead to a better assessment of readthrough compounds, the overall issue of limited drug efficacy requires further attention. The prime reason for this is the small amount of PTC-harboring mRNA are a result of NMD. A potential strategy to enlarge the small pool of PTC-harboring mRNAs will be to combine readthrough compounds with NMD inhibitors. Preclinically, it has been shown that such combinations can result in sufficiently high mRNA levels to increase protein activity [64,87]. A challenge is that NMD, like ribosomal translation control, is a pivotal cellular quality control process, and inhibition is associated with toxicity [88]. Inhibition of SMG1 via SMG1i has been studied most, and has shown large potential in preclinical studies, but high concentrations are associated with *in vitro* cellular toxicity, and this is probably the reason why SMG1i has not yet entered clinical trials. Optimization of the therapeutic window of NMD inhibitors will be of great value for their use in enhancing the efficacy of readthrough compounds. Inhibitors of other elements of the NMD machinery have also been proposed, such as vidaza (5-azacytidine, a cytidine analog), that is FDA-approved for the treatment of myelodysplastic syndrome and myeloid leukemia, and that was subsequently shown to additionally inhibit NMD in an indirect, MYC-dependent fashion [89,90]. However, vidaza therapy is associated with severe side effects and was shown to be ineffective in a previous study by our group and others [64,87]. Investigation of the potential inhibition of other proteins involved in the NMD pathway is ongoing, underlined by a recent study in which SMG6 was found to be specifically involved in CFTR degradation [91]. In addition to NMD inhibitors, readthrough compounds can be combined with drugs that enhance protein activity. CF research has yielded several drugs that enhance cellular CFTR activity, most notably amplifiers, modulators, and potentiators [92,93]. However, clinical trials on the combination of ataluren and CFTR potentiator ivacaftor did not result in positive clinical outcomes, and preliminary results of the combination therapy consisting of ELX-02 and ivacaftor indicate a similar lack of synergy.

In addition, a favorable therapeutic window is a main requirement for success of readthrough compounds as monotherapy. ELX-02 and ataluren were previously shown to have poor pharmacokinetic characteristics, indicated by their peak plasma concentrations after 2 h and rapid excretion [94]. Preliminary results of the latest trial on ELX-02 indicate similar pharmacokinetic challenges [83]. Steady-state drug levels in patients from this trial were considerably lower than the concentrations at which ELX-02 had been tested preclinically (2 μM vs. 80–160 μM) [16,63]. A biochemistry-centered drug development pipeline could aid in improving this essential pharmacokinetics–safety balance.

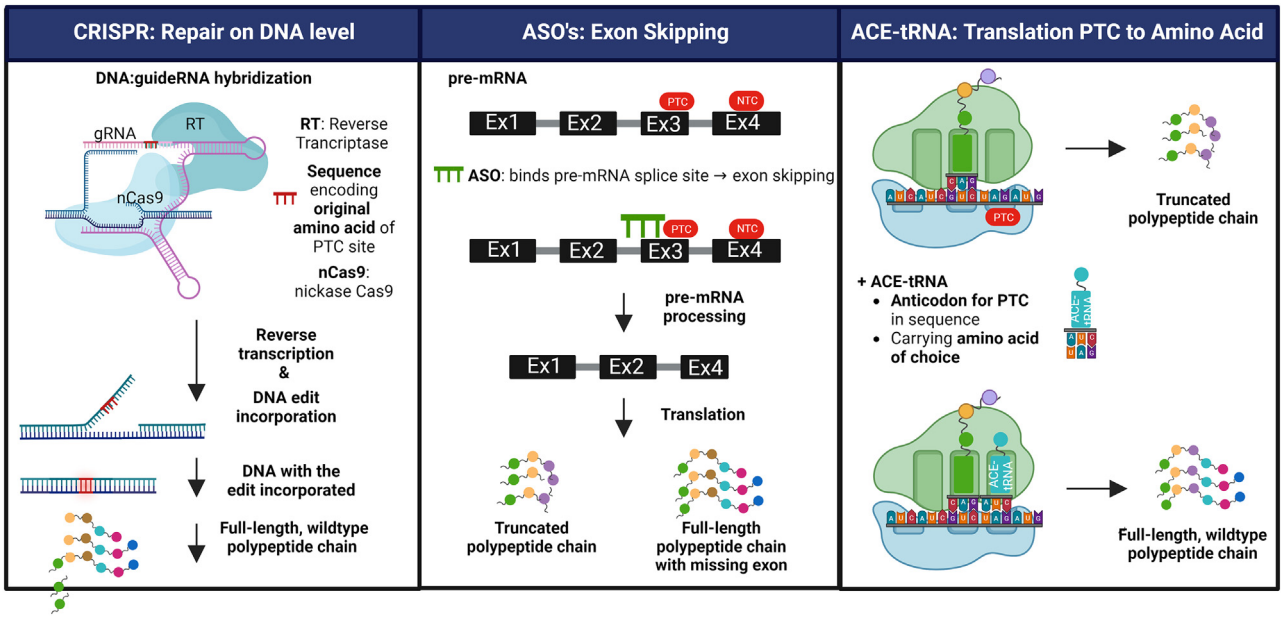

Figure 5. Non-small molecule readthrough strategies. Abbreviations: ACE-tRNA, anticodon-engineered tRNA; ASO, antisense oligonucleotide; Ex, exon; gRNA, guide RNA; NTC, natural termination codon; PTC, premature termination codon.

Finally, the field of readthrough biology is continuing the exploration of readthrough compounds with novel and potent MoAs to improve the therapeutic window and increase clinical efficacy. DAP, an inhibitor of the $tRNA^{Trp}$ modifier FTSJ1, is an example of a compound with a well-understood, potent MoA that we believe holds great potential. Interestingly, recent work on NV848/NV914 and NV930 indicates that these ataluren derivatives act in a similar manner to DAP [58]. Because both compounds have yielded promising preclinical results in relevant model systems, as well as showing a favorable safety profile in animal models [57,95], the assessment of NV848/NV914/NV930 and DAP in a clinical trial setting will be followed with great interest. Aside from this, their MoAs highlight the potential of discovering inhibitors of other tRNA-modifying proteins. Finally, multiple alternative strategies such as CRISPR technology [96,97], antisense oligonucleotide (ASO)-based strategies [98], and **anticodon-engineered tRNA (ACE-tRNA)** technology [99,100] (Figure 5) to rescue PTC mutations did not fall within the scope of this review but are worthy of further study.

In this review we have addressed several hurdles that require attention to bridge the translational gap in readthrough research (see Outstanding questions and Figure 2). In brief, it can be beneficial to prioritize preclinical assays and models that accurately recapitulate the disease and correlate with clinical outcome measures. In a clinical setting, trial standardization is pivotal. These improvements, together with a better understanding of readthrough biology and the underlying exploitable mechanisms, will be essential to make sense of nonsense mutation therapy.

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Declaration of interests

J.M.B. reports personal fees from Vertex Pharmaceuticals, Proteostasis Therapeutics, Eloxx Pharmaceuticals, Teva Pharmaceutical Industries, and Galapagos, outside the submitted work. In addition, J.M.B. has a patent related to the FIS

assay with royalties paid. C.K.v.d.E. reports grants from GSK, Nutricia, TEVA, Gilead, Vertex, ProQR, Proteostasis, Galapagos NV, and Eloxx outside the submitted work. In addition, C.K.v.d.E. has a patent 10006904 with royalties paid. The other authors declare no conflicts of interest.

Supplemental information

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